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METABOLIC FUNCTIONS OF MYOSTATIN AND GDF11

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Abstract

Myostatin is a member of the transforming growth factor β superfamily of secreted growth factors that negatively regulates skeletal muscle size. Mice null for the myostatin gene have a dramatically increased mass of individual muscles, reduced adiposity, increased insulin sensitivity, and resistance to obesity. Myostatin inhibition in adult mice also increases muscle mass which raises the possibility that anti-myostatin therapy could be a useful approach for treating diseases such as obesity or diabetes in addition to muscle wasting diseases. In this review I will describe the present state of our understanding of the role of myostatin and the closely related growth factor growth/differentiation factor 11 on metabolism.

Keywords

myostatin; activin receptor type IIB; growth/differentiation factor 11; skeletal muscle; obesity; adipocyte; metabolism

INTRODUCTION

The *myostatin* (*MSTN*) gene encodes a member of the transforming growth factor β (TGFβ) superfamily of secreted growth factors highly expressed in skeletal muscle [1]. Mice, double muscled cattle, sheep, dogs, and one child with *MSTN* loss of function genetic mutations all have dramatically increased skeletal muscle mass demonstrating that the function of the *MSTN* gene is to negatively regulate skeletal muscle growth [2]. This increased muscularity is due to hyperplasia, an increase in the absolute number of skeletal muscle fibers, and hypertrophy, an increase in the cross-sectional area of individual skeletal muscle fibers [1,3-7]. Inhibition of myostatin protein function in adult mice also increases muscle mass [8,9]. These results suggest that anti-myostatin therapy could be used as treatment for muscle wasting diseases. In addition to increased muscle mass, *Mstn*$^{-/-}$ mice and double muscled cattle have reduced fat pad mass [10-13] and are resistant to obesity and the development of insulin resistance [11,13-17]. The metabolic changes found in animals deficient for myostatin raises the possibility that myostatin inhibition could be used for treatment of metabolic diseases such as obesity and diabetes in addition to the obvious uses for treatment of muscle wasting diseases. Other recent reviews, including elsewhere in this issue, have described the potential effects of anti-myostatin therapy on a variety of muscle wasting conditions, the role of myostatin in muscle differentiation, or myostatin signaling pathways [8,9,18-21]. The effects of nutritional status and exercise on *MSTN* expression have also been reviewed previously [8,22-24] and, because they are dynamic and difficult to interpret, will not be covered here. I will therefore focus on the evidence for a metabolic role for myostatin, particularly in terms of its function in regulating adipose tissue mass and

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insulin sensitivity in normal and obesity-promoting conditions. A summary of the effects on muscle mass, muscle fiber phenotypes, fat mass, and insulin sensitivity in mice with altered myostatin levels under these conditions is presented in Table (1). In addition, the closely related TGFβ family member growth/differentiation factor 11 (GDF11) , which has some redundant functions with myostatin and may play a role in pancreatic β-cell function, will be discussed briefly. A more thorough understanding of the mechanism of action of these growth factors is likely to aid in development of new drug targets for a variety of metabolic conditions.

APPROACHES FOR MYOSTATIN INHIBITION

Like other TGFβ family members, the full-length myostatin and GDF11 proteins are proteolytically processed to form an amino-terminal propeptide and a disulfide-linked dimer of carboxy-terminal fragments. The carboxy-terminal dimer is the mature, receptor-binding molecule. Binding of the mature myostatin protein to the receptor is tightly regulated. The myostatin protein can be detected both locally in skeletal muscle and systemically in circulation in an inactive latent complex [8,9,25-27]. In circulation most myostatin is composed of the myostatin propeptide bound non-covalently to the mature region which can be activated by proteolysis of the propeptide to liberate the mature region and allow signaling through the activin type IIB receptor (ACVR2B) and at least one other unknown receptor, most likely ACVR2 [9,28-30]. Other proteins have been also been found bound to the mature myostatin dimer or the full-length unprocessed myostatin dimer. In skeletal muscle, latent TGFβ-binding protein-3 maintains full-length myostatin in an inactive complex in the extracellular matrix [31]. Follistatin (FST) is a secreted glycoprotein that can bind myostatin and inhibit its interaction with ACVR2B [8,9]. The secreted protein follistatin-like 3 (FSTL3, also known as follistatin-like related gene) is bound to myostatin in serum and also inhibits myostatin receptor binding [25]. Transgenic mice overexpressing the *FST* gene or the *FSTL3* gene in skeletal muscle have increased skeletal muscle mass [28,32]. Finally, growth and differentiation factor-associated serum protein 1 (GASP1) is bound to myostatin in serum and inhibits myostatin receptor binding [25,33]. What proportion of myostatin is bound to each of these inhibitor and whether it is bound to multiple inhibitors in one inactive complex has not been determined.

Inhibition of myostatin in wild type adult mice has been carried out by intraperitoneal injection of proteins that prevent binding of myostatin to its receptors such as a mutated uncleavable propeptide, a soluble ACVR2B/Fc fusion protein, or neutralizing monoclonal antibodies [29,34-37]. Inhibitors have also been delivered by injection of a *Mstn* DNA vaccine, by adeno-associated virus (AAV) vector expressing the mutated uncleavable *MSTN propeptide*, *FST*, *FSTL3*, or *GASP1* genes, or by inducible genetic recombination of the *Mstn* gene all of which cause an increase muscle mass [38-44]. These results demonstrate that myostatin's regulation of muscle mass is not limited to a developmental role. Furthermore, injection of the soluble ACVR2B/Fc fusion protein into $Mstn^{-/-}$ mice [29] or expressing a *FST* transgene in $Mstn^{-/-}$ mice [32] yields even greater increases in skeletal muscle mass than that found in $Mstn^{-/-}$ mice. This increase in mass over that found with deletion of the *Mstn* gene alone demonstrates that myostatin function is redundant with at least one other TGFβ family member. The amino acid sequence of GDF11 is 90% homologous to myostatin in the carboxy-terminal mature region of the protein and, like myostatin, GDF11 receptor binding is inhibited by FST and can signal through ACVR2B [8,9,45]. Activins are also inhibited by FST and FSTL3 and signal via the ACVR2 and ACVR2B receptors [46]. It is important to remember that some of the methods used to inhibit myostatin function in adults potentially also inhibit activin signaling and, in particular, GDF11 signaling, such as those methods that increase FST or dominant negative ACVR2B proteins. While the myostatin propeptide is less promiscuous, it may bind and

inhibit GDF11 as well as myostatin. I will use the term myostatin inhibition for the sake of simplicity because myostatin appears to be the family member with the greatest effect on muscle. It is possible that in other tissues the metabolic effects found are primarily due to one of the other factors with myostatin as the redundant factor.

MYOSTATIN INHIBITION AND SKELETAL MUSCLE FIBER PHENOTYPES

MSTN gene loss of function mutants

Individual muscles from *Mstn*$^{-/-}$ mice weigh approximately twice those of *Mstn*$^{+/+}$ mice [1], while those from male transgenic mice overexpressing the *MSTN* coding sequence weigh less than those from controls [47]. The weights of *Mstn*$^{+/-}$ muscles are intermediate demonstrating dose dependency for myostatin function [7]. The increase in muscle mass in *MSTN* mutants is due to changes in muscle fibers. Skeletal muscle fibers are single multinucleate cells formed by fusion of myoblasts [48]. The increase in muscle size in *Mstn*$^{+/-}$ and *Mstn*$^{-/-}$ mutant mice compared to wild type mice is due to both hypertrophy and hyperplasia of skeletal muscle fibers [1,3,5-7,49]. Muscle fibers from double muscled cattle with mutations that are expected to inactivate or eliminate the mature receptor binding region of the myostatin protein are also hypertrophic and hyperplastic [4].

MSTN null muscles are also made up of different proportions of fiber types than wild type muscles. Skeletal muscle fibers can be classified into different types depending on their metabolic and contractile properties [50]. Fast contracting fibers fatigue quickly, contain a greater concentration of glycogen, and use mainly glycolytic metabolism for energy. Slow contracting fibers are resistant to fatigue, have more mitochondria, and use predominately oxidative metabolism for energy. *Mstn*$^{-/-}$ mice have a reduction in the absolute number of slow oxidative fibers and an increase in the absolute number of fast glycolytic fibers compared to *Mstn*$^{+/+}$ mice [5,6,49]. These changes in fiber type proportions are also found in double muscled cattle [51].

Postnatal myostatin inhibition

The phenotypic effects of the loss of myostatin signaling on muscle fiber size, number, and type are different depending on whether myostatin loss of function occurs prenatally or postnatally. Thus far, myostatin inhibition in adult mice has been shown to increase the mass of individual muscles by up to approximately 50% which is half or less than the increase in muscle mass obtained by deletion of the *Mstn* gene in mice [5,29,36,37,40-44,52]. While both hypertrophy and hyperplasia occur in animals that have *MSTN* gene deletion, hypertrophy without hyperplasia is found in mice with myostatin inhibition at any time during or after the neonatal period [5,29,36,40-44,52-54]. The hypertrophy seems to affect all fiber types, particularly the fastest and most glycolytic type, designated the IIB fibers in mice [39,41]. The fiber type shifts seen in *Mstn* null mice, however, do not occur when myostatin is inhibited in normal adult animals as measured by myosin heavy chain gene or protein expression [5,41,42,44] or by histochemical staining for succinate dehydrogenase, a mitochondrial enzyme in the tricarboxcylic acid cycle [40]. Thus, both the hyperplasia and the increased glycolytic fiber number in skeletal muscle from *MSTN* null animals seem to be a consequence of the prenatal and or perinatal loss of myostatin function. Treatment of a mouse model of muscular dystrophy, the *mdx* mouse, with an AAV expressing a mutated uncleavable *Mstn propeptide*, however, increased the percent of fast glycolytic type fibers compared to untreated *mdx* mice [42]. This result suggests a possible role for myostatin in fiber type regulation under conditions of enhanced regeneration although an AAV expressing *FST* in *mdx* mice does not increase the number of glycolytic fibers as measured by succinate dehydrogenase staining intensity [40].

The metabolic studies discussed below have been carried out mainly with *Mstn*$^{-/-}$ mice. Of particular relevance to the adult biological role of myostatin as well as to potential clinical uses of myostatin inhibitors, some recent metabolic studies have been reported using mice treated with injectable myostatin inhibitors. If certain metabolic phenotypes found in *Mstn*$^{-/-}$ mice are dependent upon hyperplasia, an increase in glycolytic fiber types, or a doubling of muscle mass, treatment of adult mice with myostatin inhibitors may yield very different results. Furthermore, the metabolic consequences, if any, of an increase in muscle mass caused by hypertrophy alone versus that caused by hyperplasia with or without hypertrophy are, to my knowledge, largely unexplored.

ADIPOSITY AND INSULIN SENSITIVITY ON STANDARD DIETS

Body composition in animals with genetic *MSTN* inhibition

It is by now well established that *MSTN* null animals have reduced adiposity compared to nonmutants. *Mstn*$^{-/-}$ mice have lower absolute fat mass than *Mstn*$^{+/+}$ mice, an effect that becomes exacerbated with aging [10,11,13]. This age-dependent difference in adipose tissue deposition is also true of double muscled cattle [12]. Mice expressing a dominant negative murine *Acvr2b* transgene specifically in skeletal muscle also have increased muscle mass and reduced body fat [14]. Circulating levels of the adipose tissue-derived hormone leptin are reduced in *Mstn*$^{-/-}$ mice compared to *Mstn*$^{+/+}$ mice reflecting their reduced adiposity [10,11,14]. Transgenic mice overexpressing the *Mstn propeptide* under control of a muscle-specific promoter do not have as great a muscle mass increase as *Mstn* null mice and have less of a reduction in adiposity [17]. These results suggest that the effects of myostatin inhibition of adipose tissue mass may be at least partially dependent on the extent of muscle hypertrophy (see below).

Insulin sensitivity on a standard diet

Resistance to the hormone insulin is the primary attribute of type 2 non-insulin dependent diabetes mellitus. Insulin resistance is also a common feature of other pathologic conditions such as obesity and heart disease. Unlike type 1 diabetes, in which beta cells in the pancreas cannot produce enough insulin to maintain normal glycemia, type 2 diabetes is usually characterized by the production of higher than normal levels of insulin to compensate for the resistance to insulin action in target tissues such as skeletal muscle, liver, and adipose tissue. In addition to its well known role in stimulating muscle and adipocyte glucose uptake and suppressing hepatic glucose production, insulin also regulates the metabolism of fat and protein and is therefore a crucial regulator of overall energy storage and utilization. Although the initial mechanism leading to insulin resistance is hotly debated [55], insulin resistance in skeletal muscle is detectable long before overt hyperglycemia develops [56,57]. Because skeletal muscle takes up more glucose in response to insulin than any other tissue [56], improving skeletal muscle insulin sensitivity is an important target for increasing whole body sensitivity to insulin and preventing the onset of frank diabetes.

Mstn null mice have normal blood glucose and insulin levels, although insulin tends to be lower [11,13,14]. Several lines of evidence demonstrate that *Mstn*$^{-/-}$ mice have greater insulin sensitivity compared to *Mstn*$^{+/+}$ mice even when fed a standard diet. First, the mutant mice have improved regulation of blood glucose during glucose or insulin challenge (tolerance tests) [14]. Second, *Mstn*$^{-/-}$ mice require a greater infusion of glucose during a hyperinsulinemic-euglycemic clamp [14]. Clamps are performed by infusing a high insulin dose and measuring the amount of glucose that must be infused to maintain normal blood glucose levels. This increased requirement for infused glucose in response to elevated insulin demonstrates that peripheral tissues in *Mstn*$^{-/-}$ mice take up more glucose in response to insulin than in *Mstn*$^{+/+}$ mice. Third, the activation of the serine-threonine kinase Akt,

which mediates the signaling of insulin, insulin-like growth factor 1 (IGF1), and other growth factors, is greater in skeletal muscle and white and brown adipose tissue from *Mstn*$^{-/-}$ mice compared to *Mstn*$^{+/+}$ mice in response to insulin injection in vivo [14] indicating enhanced insulin signaling. The latter observation also suggests that these improvements in whole body insulin sensitivity in *Mstn*$^{-/-}$ mice are not due solely to an increase in muscle mass providing more peripheral sites for glucose uptake. IGF1-induced Akt activation is inhibited by myostatin in skeletal muscle cells [58,59], but a role for myostatin in the inhibition of insulin-induced activation of Akt has not yet been demonstrated. In addition, a direct effect of myostatin on insulin-stimulated or basal glucose uptake in skeletal muscle has not been described. Some contradictory results of the effect of myostatin treatment in the absence of insulin on glucose uptake in the placenta, however, have been reported. Myostatin inhibits glucose uptake in BeWo cells, a choriocarcinoma placental cell line [60]. In contrast, myostatin treatment increases glucose uptake in human placental extracts [61].

Lipid profile

Along with reduced adiposity and improved insulin sensitivity, the lipid profile in *MSTN* null animals is improved. Adult male *Mstn*$^{-/-}$ mice have significantly lower serum cholesterol and triglyceride levels compared to *Mstn*$^{+/+}$ mice [11,14]. Muscle from cattle homozygous for a *MSTN* null allele also has reduced lipid content although it is was not determined whether this is caused by reduced intermuscular adipocyte size or number, or by reduced intramyocellular lipid [62]. The concentration of hepatic triglycerides is also decreased in *Mstn*$^{-/-}$ mice compared to *Mstn*$^{+/+}$ mice [14]. Elevation of hepatic or intramyocellular lipid is a risk factor for insulin resistance [57], so these results are consistent with increased insulin sensitivity in *MSTN* null animals.

Effects of altering myostatin levels postnatally

Studies examining changes adipose tissue mass using inhibition or ectopic expression of myostatin in adult mice fed a standard diet have yielded contradictory conclusions. Two reports describing the effects on fat pad mass of direct injection of myostatin into healthy adult mice for up to 21 days gave conflicting results with one claiming reduced fat pad mass compared to PBS injected mice [63] and the other claiming no effect on fat pad mass [64]. Systemic exposure to chronic high levels of myostatin by injection of a Chinese hamster ovary cell line engineered to stably express *Mstn* causes rapid muscle and adipose tissue wasting without a decrease in food intake [63]. This method resulted in supraphysiological levels of myostatin and may therefore not be relevant to understanding the role for myostatin in fat deposition under less extreme conditions.

Experiments with myostatin inhibition in adult mice have also produced differing conclusions. Benny Klimek et al found increased abdominal fat pad weight in athymic *nude* mice injected with a CHO cell line engineered to secrete the soluble ACVR2B/Fc fusion protein [65]. The interpretation of these results is complicated, however, by the endocrine defects found in adult *nude* mice. Injection of a DNA vaccine against myostatin causes an increase in muscle mass without a corresponding decrease in abdominal fat pad mass [43]. Four weeks of treatment of healthy adult mice with the soluble receptor or the JA16 neutralizing monoclonal antibody does not affect fat mass in mice fed a standard diet, although muscle is clearly hypertrophied [36,66]. Interestingly, in mice treated with the soluble receptor for 10 weeks and fed standard diet, fat mass was significantly lower than in vehicle-treated mice and appeared to be even lower than in mice treated for 4 weeks [66]. Concomitant with the improvement in adiposity, hyperinsulinemic-euglycemic clamp analysis showed soluble receptor-treated mice also had an improvement in insulin sensitivity and increased muscle glucose uptake after 10 weeks, but not 4 weeks, of soluble receptor

treatment [66]. Taken together, these results suggest that the effects of myostatin inhibition on metabolism may be subtle but have cumulative effects on adiposity in the long-term.

EFFECTS ON ADIPOSITY AND INSULIN SENSITIVITY WITH INCREASED MUSCLE MASS BY MYOSTATIN INHIBITION IN OBESITY

Mstn null crosses to genetic models of obesity

The *Mstn* null allele has been crossed into two mouse models of obesity and insulin resistance. *Agouti lethal yellow* mice (A^y/a) have a dominant mutation that causes ectopic expression of the agouti protein which antagonizes the melanocortin 4 receptor to increase food intake and fuel efficiency [67]. These mice have adult onset obesity, hyperinsulinemia, and insulin resistance [67]. A^y/a, $Mstn^{-/-}$ double mutant mice have lower adipose tissue weights, improved fasting glucose, and improved glucose tolerance compared to A^y/a mice [11]. The *Mstn* null allele has also been crossed into the *ob*/*ob* mouse. Leptin, the product of the *ob* gene, is a peptide hormone with pleiotropic effects produced by adipocytes that, among other functions, regulates food intake and energy expenditure [68]. *ob*/*ob* mutant mice have increased food intake and reduced energy expenditure resulting in severe obesity. *ob*/*ob*, $Mstn^{-/-}$ double mutant mice have a mild decrease in fat pad mass and reduced hyperglycemia in early adulthood [11].

Diet-induced obesity in *Mstn* null and muscle-specific transgenics

Mice with deletion of the *Mstn* gene are also resistant to the effects of a high-fat diet. *Mstn* null mice gain less weight on high-fat diet than $Mstn^{+/+}$ mice [13-16]. This is also true in transgenic mice with a loss of myostatin signaling in muscle but not in adipose tissue: Mice overexpressing the *Mstn propeptide* or a dominant negative *Acvr2b* transgene specifically in skeletal muscle have increased muscle mass and are resistant to weight gain on a high-fat diet compared to non-transgenic mice [14,17]. When given at weaning, a high-fat diet actually further increases muscle mass in *Mstn* mutant mice relative to a standard diet [13,69]. This diet effect on muscle mass was not found in wild type mice.

Other metabolic consequences of diet-induced obesity are also ameliorated by myostatin inhibition. Serum insulin, leptin, cholesterol, and triglycerides are lower in *Mstn* mutant mice fed a high-fat diet than non-mutant mice fed a high-fat diet [13-15]. Insulin sensitivity and glucose metabolism, as measured by glucose and insulin tolerance tests, are also improved in *Mstn* null mice compared to $Mstn^{+/+}$ mice when fed a high-fat diet [13,14,17]. Hyperinsulinemic-euglycemic clamp experiments demonstrate that *Mstn* null mice fed a high-fat diet have greater whole body insulin sensitivity as shown by the higher glucose infusion rate required to maintain euglycemia compared to heterozygous littermates [13]. The glucose disposal rate, which is largely determined by insulin-stimulated skeletal muscle glucose uptake, is significantly higher in *Mstn* mutants than controls fed a high-fat diet [13]. This improved insulin sensitivity is also seen in mice fed a high-fat diet that are deleted for the *Mstn* gene and the *low density lipoprotein receptor* (*Ldlr*) gene [16]. In addition to a greater glucose infusion rate, $Mstn^{-/-}$, $Ldlr^{-/-}$ mice have greater glucose uptake into the quadriceps muscle during the clamp demonstrating that the muscle is more insulin sensitive by weight than muscle from $Mstn^{+/+}$, $Ldlr^{-/-}$ mice [16]. These experiments demonstrate that peripheral insulin sensitivity is increased in *Mstn* mutants compared to control mice when fed a high-fat diet.

Chronic inflammation is associated with obesity [70]. *Mstn* null mice fed a high-fat diet have reduced expression of proinflammatory cytokines in abdominal adipose tissue and in skeletal muscle, but not in liver, compared to heterozygous mice fed a high-fat diet [13]. Plasma tumor necrosis factor α (TNFα) levels are also lower in high-fat diet fed *Mstn*

mutants compared to high-fat diet-fed heterozygous controls [13]. *Mstn* null mice are also protected against high-fat diet-induced hepatic steatosis as measured by reduced hepatic triglycerides [13,14]. Liver from mutant mice fed a high-fat diet has dramatically lower levels of serine-phosphorylated insulin receptor substrate 1 and higher levels of insulin-stimulated phosphorylated Akt, both of which indicate enhanced insulin signaling compared to controls [13].

Another important pathology associated with obesity is atherosclerosis. The mouse is highly resistant to diet-induced atherosclerosis, so genetic manipulation coupled with high-fat diet feeding have been employed to create appropriate mouse models. One such model, the $Ldlr^{-/-}$ mouse, has been crossed to the $Mstn^{-/-}$ mouse to generate double mutants to study the effects of *Mstn* deletion on the development of an atherogenic lipid profile and atheromatous lesions induced by a high-fat diet [16]. $Mstn^{-/-}$, $Ldlr^{-/-}$ mice are muscular and resistant to diet-induced obesity. Double mutants also have lower plasma free fatty acids, triglycerides, and cholesterol, have reduced hepatic steatosis, and have increased insulin sensitivity compared to $Mstn^{+/+}$, $Ldlr^{-/-}$ mice. The number and area of diet-induced artherosclerotic plaques are consequently significantly lower in aortae from $Mstn^{-/-}$, $Ldlr^{-/-}$ mice than in aortae from $Mstn^{+/+}$, $Ldlr^{-/-}$ mice. Thus, *Mstn* gene deletion in mice prevents the development of the major pathological consequences of obesity.

Postnatal myostatin inhibition in obesity models

A recent study describes the postnatal inhibition of myostatin using a soluble ACVR2B/Fc fusion protein in a diet-induced obesity model [66]. When given simultaneous with placement on a high-fat diet, the soluble receptor increases lean mass by 4 weeks post-treatment without preventing the diet-induced increase in adiposity. There were, however, signs that myostatin inhibition had an effect on adipose and liver metabolism after only 4 weeks. The concentration of adiponectin in serum, a fat-specific hormone that promotes insulin sensitivity, increased in mice treated with the soluble receptor but only when fed a high-fat diet and not when fed a standard diet. Although glucose uptake was identical to non-treated mice, hepatic glucose production during the clamp was significantly lower in soluble ACVR2B/Fc-treated mice fed a high-fat diet. More dramatic effects were seen in soluble receptor-injected mice after 10 weeks of treatment and high-fat diet feeding. The soluble receptor appeared to prevent further gain in fat mass caused by high-fat diet feeding which resulted in approximately half as much body fat as untreated mice by this time point. After 10 weeks on the diet, mice receiving the soluble receptor treatment also had better insulin sensitivity compared to vehicle-treated mice as measured by the hyperinsulinemiceuglycemic clamp test.

Can inhibition of myostatin reduce adipose tissue mass after the onset of obesity? Stolz et al injected 60 mg/kg body weight of the JA16 antibody for 9 weeks into adult *db*/*db* mice, an obesity model caused by mutation of the gene encoding the leptin receptor [64]. Although this treatment increased muscle mass, it did not reduce fat pad mass. Similar results were obtained using a different anti-myostatin neutralizing antibody, PF-879, to treat *ob*/*ob* mice for 6 weeks [34]. It is possible that a greater increase in muscle mass is required to see an effect on adipose tissue mass with myostatin inhibition in such severe obesity models. Alternatively, myostatin inhibition may only be efficacious in reducing adiposity in animals with mild obesity. These results together with those from the time course using soluble receptor injections simultaneous with high-fat diet feeding described above [66] indicate that greater than 4 weeks of treatment will be needed before a conclusion can be drawn about the effects of myostatin inhibition on adipose mass. Although adipose mass was unaffected, PF-879 treatment reduced fasting and non-fasting blood glucose and improved glucose tolerance, but not insulin tolerance, in *ob*/*ob* mice [34]. Taken together, experiments carried out by injecting myostatin inhibitors into mouse models of obesity suggest that anti-

myostatin therapy may be more useful for improving glucose metabolism than for treating obesity.

DIRECT VERSUS INDIRECT EFFECTS ON ADIPOSE TISSUE

Expression in adipose tissue

The genes for *MSTN,* its receptor *ACVR2B,* and its inhibitors *FST* and *FSTL3* are expressed in adipose tissue [1,71-74]. These expression patterns have been analyzed in detail in mice. *Mstn* mRNA expression in adipose tissue is very low, at levels that are approximately 50- to 100-fold lower than in skeletal muscle [71]. *Acvr2b* is also expressed in adipose tissue at lower levels than in skeletal muscle [64,71]. *Fstl3* expression is considerably higher than *Mstn* and *Acvr2b* expression in adipose tissue, particularly in the visceral adipose tissue compartment, and is comparable to *Fstl3* expression in skeletal muscle [71]. These data would seem to indicate that the level of myostatin signaling in adipose might normally be quite low. Within the subcutaneous fat pad, the expression levels of the *Mstn*, *Acvr2b*, and *Fstl3* genes are greater in the mature adipocyte fraction than in the stromal/vascular fraction [71] which contains endothelial progenitors, monocytes, macrophages, T regulatory cells, committed preadipocytes, and mesenchymal stem cells [75]. *Fst*, however, is predominantly expressed in the stromal-vascular fraction [72].

In vitro effects of myostatin

Some interesting experiments have been carried out with cell lines that are at different stages of development of the adipogenic lineage. C3H 10T1/2 mesenchymal cells are multipotent and can differentiate into the chondrogenic, myogenic, or adipogenic lineages. Myostatin inhibits the induction of adipogenic differentiation of C3H 10T1/2 cells by bone morphogenetic protein 7 (BMP7) due to competition with BMP7 for ACVR2B receptor binding [30]. In contrast, myostatin promotes differentiation toward the adipogenic pathway in C3H 10T1/2 cells induced to differentiate by other means. For example, myostatin treatment of these cells promotes adipogenic differentiation induced by 5'-azacytidine, although high concentrations of myostatin are required [76]. When C3H 10T1/2 cells are treated with a cocktail of the glucocorticoid dexamethasone, insulin, and 3-isobutyl-1-methylxanthine (IBMX), *Mstn* expression is strongly induced specifically by the glucocorticoid [77]. In fact, myostatin protein can substitute for dexamethasone to induce differentiation in these cells. The myostatin, insulin, and IBMX-treated cells do not differentiate to the same extent as with glucocorticoid treatment, yielding instead small immature adipocytes. These myostatin-induced cells, however, are more insulin sensitive. C3H 10T1/2 cells differentiated with myostatin, insulin, and IBMX have greater insulin-stimulated glucose uptake compared to cells differentiated with dexamethasone, insulin, and IBMX [77]. Myostatin also has a role in preadipocytes differentiation but has different effects at this stage of adipogenesis. A *MSTN* promoter construct is activated by inducers of adipogenesis such as CAAT/enhancer binding protein α, peroxisome proliferator activated receptor γ or sterol regulatory element-binding protein 1c in 3T3-L1 preadipocytes [71]. In contrast to its effect on mesenchymal cells, myostatin inhibits the differentiation of 3T3-L1 preadipocytes [5,30,63,78] and bovine preadipocytes [74] in vitro. Taken together, these results suggest that myostatin promotes a cell fate decision toward the adipocyte pathway in uncommitted cells but inhibits differentiation in committed preadipocytes.

Adipose-specific manipulation of myostatin levels on standard diets

Analyses of transgenic mice with increased or decreased muscle mass demonstrate that altered skeletal muscle mass alone can affect adipose tissue mass. Transgenic mice expressing *c-Ski* under control of the murine sarcoma virus long terminal repeat have increased lean mass and reduced body fat [79,80]. Similarly, skeletal muscle-specific *IGF1*

transgenic mice and pigs [81,82] and skeletal muscle-specific constitutively active *Akt* transgenic mice [83,84] have reduced adipose tissue mass compared to non-transgenic controls. In addition, male transgenic mice overexpressing *Mstn* specifically in skeletal muscle have reduced muscle mass and increased epididymal fat pad mass [47]. In general, the degree of the effect on adiposity seems to inversely correlate with the magnitude of muscle hypertrophy or atrophy and an earlier age of onset of muscle mass alteration.

These observations demonstrate that analysis of the effects of myostatin on adipose tissue mass is confounded by the presence of muscle hypertrophy in *Mstn*$^{-/-}$ mice. Therefore, my laboratory made a transgenic line of mice overexpressing a dominant negative *Acvr2b* transgene using the *aP2* (fatty acid binding protein 4) promoter to inhibit myostatin signaling specifically in adipocytes [14]. We found no difference in body composition between transgenic mice and littermate controls fed either a standard or a high-fat diet. Expression of the same dominant negative *Acvr2b* construct under the control of a muscle-specific promoter, however, phenocopies the body composition and resistance to diet-induced obesity found in *Mstn*$^{-/-}$ mice [14]. These data suggest that myostatin does not directly regulate fat mass, and that the effects of myostatin inhibition in reducing adipose tissue mass in *Mstn*$^{-/-}$ mice are indirectly caused by the increase in muscle mass.

This approach, using the *aP2* promoter to drive expression of a membrane-bound transgene, does not address whether the cell fate decision is affected because the *aP2* gene is expressed at later stages of adipogenesis. Feldman et al [77] reported a transgenic mouse line overexpressing *Mstn* from an *aP2* promoter construct. Because myostatin is a secreted protein, myostatin signaling might not be cell-autonomous in this line. In other words, this transgenic line might be expected to have increased myostatin signaling in both the adipocytes expressing the transgene and in any neighboring preadipocytes or mesenchymal stem cells that are most likely not expressing the transgene. These fat-specific *Mstn* transgenic mice have small, immature adipocytes similar to those induced by treatment of mesenchymal cells with myostatin, insulin, and IBMX in vitro. This observation is consistent with a role for myostatin in promoting the cell fate decision of stem cells and inhibiting the terminal differentiation of preadipocytes. Although the mice have smaller adipocytes and lower overall body weight, surprisingly, they have normal body composition. Glucose tolerance is also improved in these mice compared to nontransgenic controls. Small adipocytes have a lower fatty acid flux and a more favorable cytokine secretion profile which favor higher insulin sensitivity than large adipocytes [85]. Consistent with this size difference, adipocytes isolated from transgenic mice overexpressing the *Mstn* gene in adipocytes have an increase in insulin-stimulated glucose uptake compared to adipocytes from non-transgenic mice [77]. It is interesting that the reduced adipocyte size and improvement in insulin sensitivity in these mice did not cause a reduction in adiposity in transgenic mice on a standard diet. A resistance to weight gain on a normal diet may, however, be evident in these mice with increasing age when mice normally accumulate more adipose tissue.

Adipose-specific manipulation of myostatin levels on high-fat diets

Although they have normal body composition on a standard diet, fat-specific *Mstn* transgenic mice do not gain as much weight when placed on a high-fat diet as non-transgenic mice [77]. They also have better glucose tolerance, lower fasting glucose, insulin, and triglycerides. This resistance to the effects of diet-induced obesity is apparently because of an increased metabolic rate (measured in mice on a standard diet) due to the smaller adipocytes. Expression of an adipose-specific dominant negative *Acvr2b* gene, in contrast, does not prevent weight gain or the development of insulin resistance caused by high-fat diet feeding [14].

These data indicate that myostatin can directly affect adipose tissue size under some conditions, but that it is overexpression, rather than underexpression, that inhibits fat accumulation. Specifically, resistance to diet-induced obesity can be achieved by either the reduction of myostatin signaling in muscle or the increase in myostatin signaling in adipose tissue.

Possible functions in adipose tissue

Although transgenic mice carrying a fat-specific dominant negative *Acvr2b* transgene had normal body composition, they did tend to become larger than non-transgenic mice with age due to an increase in both lean and fat mass for unknown reasons suggesting some change in metabolism (ACM unpublished data). We also found that serum free fatty acid levels were significantly lower in these transgenic mice when fed a standard diet [14], although myostatin does not increase the rate of lipolysis of adipocytes in vitro [64]. Serum resistin was also significantly higher in fat-specific dominant negative *Acvr2b* transgenic mice fed a high-fat diet even though fat mass was not increased [14]. This indicates that there may be effects of myostatin signaling on adipocytes that regulate adipocyte function rather than size. One possibility is the production of the adipokine adiponectin. Adiponectin promotes insulin sensitizing mechanisms such as the inhibition of hepatic gluconeogenesis and increased fatty acid oxidation in liver and muscle [86-88]. Transgenic mice overexpressing the *MSTN propeptide* in skeletal muscle have higher expression of *adiponectin* mRNA in epididymal fat pads than non-transgenic mice on both standard and high-fat diets [89], although this could be due to the fact that serum adiponectin levels are normally inversely correlated with adipose mass and these mice are lean. Surprisingly, serum adiponectin increases with high-fat diet feeding in these transgenic mice even though adipose tissue mass does not change [17]. Serum adiponectin also increases in soluble ACVR2B/Fc-treated mice fed a high-fat diet after 4, but not 10, weeks of treatment and diet [66]. Injection of a neutralizing anti-myostatin antibody into *ob*/*ob* mice also increases *adiponectin* mRNA expression in adipose tissue [34]. Whether myostatin regulates *adiponectin* gene expression directly remains to be determined. In summary, the majority of experiments thus far suggest that myostatin signaling in adipocytes does not directly regulate adipose tissue mass under standard laboratory conditions or with high-fat diet feeding in mice, but there are some data indicating a role for myostatin in regulating adipocyte metabolism.

MYOSTATIN AND INSULIN RESISTANCE INDEPENDENT OF MUSCLE MASS

Expression in obesity and diabetes

There is some evidence that *Mstn* expression in skeletal muscle is positively correlated with insulin resistance in muscle regardless of muscle size. *Mstn* gene expression in tibialis anterior muscle is increased in *ob*/*ob* mice compared to wild type mice [71]. Tibialis muscle weight is decreased in these mice which might be expected for a muscle with increased *Mstn* expression. *Mstn* expression in the tibialis muscle from high-fat diet fed non-mutant mice is also increased, however, without a change in muscle mass compared to standard diet-fed mice [71]. In cultured myotubes from extremely obese patients with an average body mass index (BMI) of 49 kg/m^2, myostatin protein is secreted at higher levels compared to myotubes from less obese (BMI range of 25 to < 40 kg/m^2) and normal body weight subjects [90]. There is no information on lean mass, but this patient group had significantly higher homeostasis model assessment levels (HOMA), a calculation obtained from fasting plasma insulin and glucose concentrations. Higher HOMA levels indicate these patients are likely to have insulin resistance in addition to higher body weight. Furthermore, the expression of the *MSTN* gene in muscle from diabetic, morbidly obese patients is reduced after gastric bypass surgery when body weight, insulin resistance, fat mass, and lean mass

are reduced [91,92]. *MSTN* mRNA expression is also elevated 1.76-fold in the vastus lateralis muscle of first degree relatives of type 2 diabetes patients compared to healthy controls matched for fat free mass and BMI [93]. Although not overweight, these subjects had demonstrable insulin resistance measured during a hyperinsulinemic-euglycemic clamp. Taken together, these data show that expression levels of the *MSTN* gene do not necessarily correlate with muscle mass or lean mass and suggest that myostatin could play a more direct role in skeletal muscle metabolism in addition to an indirect affect on metabolism by regulating muscle size.

Gene expression data also suggest that the levels myostatin signaling pathway genes are altered in adipose tissue in response to obesity. The expression levels of *Mstn* and *Acvr2b* are 50- to 100-fold higher in adipose tissue from *ob*/*ob* mice compared to normal weight mice [71]. *Fstl3* expression is increased in the subcutaneous adipose tissue and decreased in the visceral adipose tissue from *ob*/*ob* mice compared to wild type mice [71]. *Mstn* expression, but not *Acvr2b* or *Fstl3* expression, in subcutaneous adipose tissue also increases with increasing diet-induced obesity in mice [71]. Although *MSTN* expression was not determined, *FST* gene expression is slightly higher in subcutaneous adipose tissue from obese women [72]. *Fstl3* knockout mice have reduced visceral fat pad depots although they have similar body composition compared to wild type mice [94], suggesting that the FSTL3 protein plays a role in visceral adipose tissue. In summary, the expression data suggest a possible role for myostatin signaling in adipose tissue from obese individuals, but there is as yet no data correlating increased *MSTN* expression or increased myostatin signaling with insulin resistance per se in adipose tissue. Given the reduced adipocyte size and increased insulin sensitivity found in mice overexpressing a *Mstn* transgene specifically in adipose tissue [77], it would be informative to be able to compare the adipocyte size and level of myostatin signaling in insulin resistant mice. Perhaps there is a dose-dependent effect on adipocyte size, or adipocytes of different sizes respond differently to increased myostatin.

Myostatin regulation of insulin sensitivity

To date, the strongest evidence that myostatin regulates insulin sensitivity by a mechanism other than affecting muscle mass comes from the reintroduction of the myostatin protein into *Mstn* null mice. Wilkes et al [13] fed control and *Mstn* null mice a high-fat diet which caused the control mice, but not the *Mstn* null mice, to became insulin resistant as measured by insulin tolerance tests. These insulin sensitive *Mstn* null mice were then injected with either placebo or myostatin protein for only 5 days. Myostatin injection significantly worsened the performance of *Mstn* null mice on the insulin tolerance test compared to the placebo-injected *Mstn* null mice without any apparent change in body weight or lean mass. In fact, the insulin tolerance tests from *Mstn* null mice injected with myostatin and heterozygous mice not injected with myostatin were indistinguishable. Plasma TNFα levels increased in response to myostatin injections indicating a potential mechanistic link between myostatin levels and insulin resistance. This effect may only be measureable in mice with reduced myostatin levels, which are likely more sensitive to myostatin, and/or mice in which insulin sensitivity is already challenged such as with high-fat diet feeding. Nevertheless, this is the only functional experiment thus far to my knowledge that detected a change in insulin sensitivity without a change in muscle size after acutely altering myostatin levels.

Regulation of *MSTN* expression by glucocorticoids

The promoter of the *MSTN* gene contains glucocorticoid response elements, thyroid response elements, and an androgen response element suggesting that these hormones may regulate *MSTN* gene expression [95]. The glucocorticoid dexamethasone induces *Mstn* expression in the C2C12 myoblast cell line [95], in muscle in vivo [96], and in C3H 10T-1/2 cells upon induction of adipogenesis [77]. In a reporter assay using the sheep *MSTN*

promoter in C2C12 myoblasts, transcriptional activity is reduced by approximately 30% and dexamethasone-stimulated activity is abolished when glucocorticoid response elements are mutated [97]. Pharmacologic treatment with glucocorticoids for immune suppression can cause side effects such as muscle wasting [98]. Wasting and *Mstn* gene expression in skeletal muscle are induced by dexamethasone injection in rats [96], both of which are prevented by glutamine treatment [99]. Furthermore, $Mstn^{-/-}$ mice are protected against dexamethasone-induced muscle wasting [100] suggesting anti-myostatin therapy may be an effective treatment to halt muscle wasting in patients prescribed immunosuppressive glucocorticoids. Elevated local or systemic glucocorticoid levels can also cause insulin resistance, hepatic steatosis, and dyslipidemia [98]. It is pure speculation, but this raises the possibility that the higher levels of *MSTN* gene expression described above in adipose and muscle from mice and humans susceptible to insulin resistance due to obesity or predisposition is caused by increased glucocorticoid levels in these tissues. It has yet to be shown whether myostatin inhibition can block any of these glucocorticoid-induced metabolic defects analogous to its effects on glucocorticoid-induced muscle wasting.

In other species, glucocorticoids seem to have the opposite effect on *MSTN* gene expression. Subtherapeutic oral doses of glucocorticoids added to feed significantly repress *MSTN* expression in bovine biceps muscle [101]. Similarly, in tilapia fish larvae, *MSTN* expression is down-regulated by cortisol in the tank water in as little as 3 hours [102]. Glucocorticoids are known to increase in response to stress in fish as well as mammals [103], and *MSTN* expression is down-regulated by overcrowding stress in zebrafish [104]. These different responses of *MSTN* gene expression to glucocorticoid exposure could be due to species differences. More interestingly, although it is difficult to compare in vivo dosage received in bovine feed and fish tank water to that received by subcutaneous injection into the mouse, these studies raise the intriguing possibility that the effects of exogenous glucocorticoids on *MSTN* gene expression may be different depending on background cortisol/corticosterone levels which vary depending on such factors as stress, fasting state, or time of day.

POTENTIAL MECHANISMS FOR REDUCED ADIPOSITY AND RESISTANCE TO OBESITY

Energy expenditure

One possible explanation for the reduced adipose tissue mass in $Mstn^{-/-}$ mice that has been considered is increased energy expenditure. Activity levels in $Mstn^{-/-}$ mice on a normal diet are not significantly higher than in $Mstn^{+/+}$ mice although these data have a large standard deviation [14]. *ob*/*ob* mice treated with a neutralizing monoclonal antibody to myostatin have increased activity, oxygen consumption normalized to body weight, and energy expenditure without a reduction in fat mass [34]. When crossed to the $Ldlr^{-/-}$ mouse and placed on a high-fat diet, $Mstn^{-/-}$, $Ldlr^{-/-}$ double mutant mice have a 38% reduction in activity compared to $Mstn^{+/+}$, $Ldlr^{-/-}$ mice but reduced adiposity [16]. Thus, activity levels do not seem to explain the changes in adipose tissue mass.

Arch et al. [105] recently published a detailed review of the difficulties in interpreting energy expenditure data in mice. The issues raised are particularly relevant for analysis of energy expenditure in $Mstn^{-/-}$ mice. Because the amount of body surface area affects heat loss, and adipose tissue is less metabolically active than lean tissue, metabolic rate data require correction for body size and body composition. How to best achieve meaningful normalization, however, is a subject of debate. By indirect calorimetry, a method allowing the measurement of the volume of O_2 (VO_2) consumed and of the volume of CO_2 produced by an individual mouse in a closed environment, $Mstn^{-/-}$ mice show an increase in VO_2 per mouse compared to $Mstn^{+/+}$ mice [11,14]. The $Mstn^{+/+}$ mice and $Mstn^{-/-}$ mice can differ

dramatically in body weight and body composition, and the magnitude of these differences change over time as the animals age. Normalizing the VO_2 consumed by *Mstn*$^{-/-}$ mice to some of the commonly used factors such as body mass, body mass to the ¾ power, lean mass, or lean mass to the ¾ power, however, yields conflicting results. For example, *Mstn*$^{-/-}$ mice have higher calculated metabolic rate per mouse or per body weight to the ¾ power, but lower metabolic rate when normalized to lean mass or lean mass to the ¾ power ([11,14], and analysis of data from same). When normalized to body weight, the metabolic rate of *Mstn*$^{-/-}$ mice is lower than that of *Mstn*$^{+/+}$ mice at younger ages when *Mstn*$^{+/+}$ mice have a relatively lower body weight [11]. In contrast, metabolic rate is higher in *Mstn*$^{-/-}$ mice compared to *Mstn*$^{+/+}$ mice at older ages when the body weights are more comparable (analysis of data from ref [14]). Furthermore, the organs that comprise lean (or fat free) mass differ in their individual metabolic rates with skeletal muscle having a relatively low metabolic rate. Because the increase in lean mass in *Mstn*$^{-/-}$ mice is due to the increase in skeletal muscle size rather than to a proportional increase in all organs comprising the lean component of body weight, normalizing to lean mass for a comparison between *Mstn*$^{+/+}$ and *Mstn*$^{-/-}$ mice underestimates the contribution of high energy usage organs in *Mstn*$^{-/-}$ mice such as the brain, kidney, and heart. Regression analysis of fat free mass versus energy expenditure, the common method for analysis of metabolic rate in humans, is difficult in mice, particularly *Mstn*$^{+/+}$ mice versus *Mstn*$^{-/-}$ mice, because the within-group (within a genotype) variance is much lower than between-group variance. The same issues of normalization arise when considering food intake data. Food intake in *Mstn*$^{-/-}$ mice is similar to *Mstn*$^{+/+}$ mice in proportion to body weight, although higher per mouse when calculated at an age in which *Mstn*$^{-/-}$ mice are 23% heavier than wild type littermates [11]. Additionally, whether these relationships between body weight and food intake are linear with aging during which the *Mstn*$^{+/+}$ mice, but not the *Mstn*$^{-/-}$ mice, develop increased adiposity is unknown. Regardless, the lack of an increase in body weight over time suggests that energy intake and energy expenditure in *Mstn*$^{-/-}$ mice must be nearly equal.

Hepatic fatty acid oxidation

Increased muscle mass can indirectly alter metabolism of liver as well as adipose tissue. Mice made obese by a high-fat diet lose weight after induction of a constitutively active *Akt* transgene expressed specifically in skeletal muscle [83]. The effect on body weight is not seen until after the development of increased muscle mass, specifically, increased fast glycolytic fiber hypertrophy. These mice have increased fatty acid oxidation in liver possibly as a response to increased glucose energy demand by the hypertrophied skeletal muscle. Similarly, mitochondria isolated from liver from *Mstn*$^{-/-}$, *Ldlr*$^{-/-}$ mice fed a high-fat diet have greater lipid oxidation in vitro compared to mitochondria isolated from liver from *Mstn*$^{+/+}$, *Ldlr*$^{-/-}$ mice fed a high-fat diet [16]. Blood ketone levels are also increased in these mice indicating that fatty acid beta oxidation is higher in double mutant mice. In contrast, blood ketone levels are not elevated in *Mstn*$^{-/-}$ mice fed a high-fat diet, and liver gene expression data do not suggest an increase in hepatic fatty acid oxidation in these mice [14]. In mice fed a standard diet, fatty acid oxidation in *Mstn*$^{-/-}$ liver homogenates is not increased compared to *Mstn*$^{+/+}$ liver nor in mice receiving injections of the JA16 antibody [64]. Thus, increased fatty acid oxidation in liver is not a consistent feature of resistance to diet-induced obesity in *Mstn* null mutants and may depend on factors such as diet composition and length of time on high-fat diet. Liver from *Mstn*$^{-/-}$ mice fed either standard or high-fat diet has reduced triglycerides [14], but this could be due to reduced need for hepatic fatty acid uptake with reduced adiposity.

GDF11 AND METABOLISM

Like myostatin, the GDF11 protein is detectable in human serum [26], signals through the ACVR2 and ACVR2B receptors [28,106], and is antagonized by FST [28,45,107-109]. The

tissue source for this circulating GDF11 is not known. The *Gdf11* gene has a broader expression pattern than the *Mstn* gene in mice: It is expressed in the pancreas, intestine, kidney, skeletal muscle, and developing nervous system, for example [45,110-114]. Deletion of the *Gdf11* gene in mice causes anterior homeotic transformation of the axial skeleton with lumbar vertebrae transformed into thoracic vertebrae [112]. Double *Mstn, Gdf11* mutants have an even greater degree of transformation in addition to other skeletal defects not found in either single mutant demonstrating redundant functions in skeletal development and patterning [6]. Skeletal muscle-specific deletion of the *Gdf11* gene in a $Mstn^{+/+}$ or $Mstn^{-/-}$ background, however, does not increase muscle mass, fiber number, or fiber type [6].

In the pancreas, in addition to skeletal transformation, changes in the proportion of pancreatic cell types have been described in newborn $Gdf11^{-/-}$ mice [115,116]. *Gdf11* is expressed in the developing pancreatic epithelium [115,116]. The number of acinar cells of the exocrine pancreas is reduced while the number of neurogenin 3+ islet progenitors is increased in $Gdf11^{-/-}$ newborns [115,116]. Two studies reach different conclusions, however, regarding the role of GDF11 in differentiation of β-cells, the islet cells that produce insulin. Harmon et al [116] found evidence of impaired maturation of β-cells, an overall reduction in β-cell mass, and an increased α-, or glucagon-producing, cell mass in mutants compared to $Gdf11^{+/+}$ newborns. Dichmann et al [115], however, found normal β-cell maturation and normal numbers of β- and α-cells in the $Gdf11^{-/-}$ pancreas. The reasons for these discrepancies are not clear.

Activin receptor signaling clearly plays a role in β-cell differentiation. Activin receptors are expressed in the developing and regenerating pancreas [117,118]. Islets from activin receptor mutants are hypoplastic [119,120], and FST inhibits β-cell maturation [118]. Inhibition of all TGFβ family signaling in adult β-cells by conditional transgenic overexpression of a gene encoding an intracellular inhibitor of TGFβ family signaling, SMAD7, causes diabetes because of a loss of insulin production [121]. Which specific family members cause this phenotype is not known. GDF11 may be a plausible candidate, but redundancy of this function seems likely given that activins are also expressed in the pancreas [117].

The *Gdf11* gene is also expressed in the gastrointestinal tract, and $Gdf11^{-/-}$ mice have malformations of the stomach [116]. $Gdf11^{-/-}$ mice die perinatally presumably due to developmental defects in kidney and palate formation [6] precluding an analysis of the role of GDF11 in the adult pancreas in these mice. An investigation of a postnatal role for GDF11 signaling in insulin synthesis or secretion or the gut response to diet, for instance, will require conditional approaches targeting the *Gdf11* gene or downstream effectors. It will also be important to keep potential GDF11 functions in mind when using investigative or therapeutic approaches for reducing myostatin function because some inhibitors will reduce the receptor binding of both proteins.

CONCLUSION

It remains to be seen which, if any, metabolic disturbances such as immune suppression by glucocorticoid therapy, the metabolic syndrome, diabetes, or obesity might be improved by anti-myostatin treatment particularly after the onset of illness. In a study of Australian men, muscle mass and strength were found to positively correlate with reduced incidence of the metabolic syndrome, a group of risk factors for cardiovascular disease that includes insulin resistance [122]. Anti-myostatin therapy may be a resistance exercise mimetic in patients unable to exercise, for example. Resistance exercise as well as endurance exercise improves diabetes in humans [123-126]. In fact, the American College of Sports Medicine recommends that diabetic patients add resistance training as part of an exercise regimen to

increase muscle mass and improve glucose metabolism [127]. Even if these experiments fail, there may still be a use for anti-myostatin therapy in maintenance of health after other therapies. Along these lines, a recent study showed that resistance exercise helped prevent weight regain after weight loss in women [128].

The lag between the muscle mass increase and the improvements in adiposity and insulin sensitivity after soluble receptor treatment in mice on high-fat diet [66] suggests that changes in skeletal muscle metabolism might be minor but result in cumulative effects. These effects eventually either cause a reduction in fat mass and increased insulin sensitivity or partially prevent the accumulation of fat and development of insulin resistance. Studies examining the role of myostatin inhibition on metabolism may therefore require more than four weeks of treatment with myostatin inhibitors in mice.

Mstn null mice have reduced adiposity due to an indirect effect on adipose mass, but there are tantalizing hints that myostatin may play a more direct role in regulating insulin sensitivity. A major question is whether any identified responses in mice with adult onset myostatin inhibition or addition are due to alteration in muscle mass or to changes in myostatin signaling or both. We know very little about the downstream effects of myostatin signaling on metabolism. Recent microarray experiments with muscle obtained from mice after myostatin inhibition during adulthood suggests there are relatively few changes at the level of gene expression in response to the loss of myostatin signaling despite increases in muscle mass [129]. Other approaches, such as proteomic or metabolomic techniques, may be more informative. Another approach is varying dosage of myostatin or its inhibitors. For example, leptin's regulation of glucose metabolism has been uncoupled from its regulation of fat mass by using low dose injections that do not affect body weight [130]. An analogous approach using low doses of myostatin antagonists or myostatin itself may allow us to begin to address these questions.

Another question is whether *Mstn*$^{-/-}$ mice have metabolic impairments. Amthor et al [3] found a disproportional decrease in mitochondria in *Mstn*$^{-/-}$ muscle, and a greater than expected number of tubular aggregates, non-specific accumulations of sarcoplasmic reticular membranes, in aging fast glycolytic fibers in *Mstn*$^{-/-}$ mice. Whether tubular aggregates are a symptom of pathological changes in *Mstn*$^{-/-}$ mice or whether they also occur after myostatin inhibition in adult animals or other species is unknown. We also do not know whether lean myostatin-deficient animals are able to adequately adapt to fasting and starvation by mobilization of glycogen, fat, and protein. Answers to these questions about knockout mice can help guide analysis of the more therapeutic approaches, but are particularly important for clinical management of people carrying *MSTN* gene mutations and the production and management of farm animals with *MSTN* loss of function mutations.

The ability of ACVR2B to bind multiple TGFβ family members also raises the possibility that treatment with the soluble receptor inhibits other ligands besides myostatin. A comparison of the efficacy of myostatin-specific inhibition versus more promiscuous inhibition will allow us to tease out which ligands play which roles. Inhibiting other ligands may be beneficial or cause unwanted side effects. Alternatively, there could also be a mechanism based on reduced competition for receptor binding. Different proportions of TGFβ family members signaling in the same cell line cause different downstream effects. For example, myostatin reduces BMP7 signaling by competing for ACVR2B receptor binding which alters the downstream signaling cascade even though they use the same type II receptor [30]. Hypothetically, in mice treated with the soluble ACVR2B/Fc, the reduction in myostatin levels would allow for increased signaling through ACVR2B or ACVR2 by another ligand whose levels are less affected by the soluble receptor.

Considerable progress has been made in the analysis of metabolic changes in mice with *MSTN* overexpression or inhibition, and the effects of adult inhibition of myostatin on metabolism are beginning to be elucidated. The field seems to be poised to soon determine whether myostatin plays a role in the progression of metabolic diseases and whether modulating myostatin levels will be therapeutically advantageous for treating a number of different conditions.

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ABBREVIATIONS

AAV	adeno associated virus
ACVR2	activin type II receptor
ACVR2B	activin type IIB receptor
aP2	fatty acid binding protein 4
BMI	body mass index
BMP	bone morphogenetic protein
FST	follistatin
FSTL3	follistatin-like 3 or follistatin-like related gene
GASP1	growth and differentiation factor-associated serum protein 1
GDF11	growth/differentiation factor 11
IBMX	3-Isobutyl-1-methylxanthine
IGF1	insulin-like growth factor 1
LDLR	low-density lipoprotein receptor
MSTN	myostatin
TGFβ	transforming growth factor β
TNFα	tumor necrosis factor α
$\boldsymbol{VO_2}$	volume of oxygen

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Table 1

Muscle and adipose phenotypes in mice with altered myostatin levels.

	MUSCLE		STANDARD DIET			HIGH-FAT DIET/GENETIC OBESITY			
	Muscle mass	**Fiber Phenotype**	**Fat Mass**	**Ins sens[a]**	**Other**	**Obesity**	**Ins sens[a]**	**Other**	**References**
DIET									
***MSTN* deletion**									
Mstn$^{-/-}$	↑	↑size ↑number ↑FG	↓	↑	↑GT	↓	↑	↑GT	[1,3,5-7,14,15,49]
Mstn$^{Ln/Ln}$	↑		↓			↓	↑	↑GT	[13]
Mstn$^{-/-}$, *Ldlr*$^{-/-}$	↑					↓	↑		[16]
Transgenic models									
Muscle-specific									
Mstn Propeptide	↑	↑size ↑number		=	=GT	↓	↑	↑GT	[17,28,131]
DN Acvr2b	↑	↑size ↑number	↓	↑	↑GT	↓	↑	↑GT	[14,28]
Mstn	↓	↓size =type	↑						[47]
Fat-specific									
DN Acvr2b	=[b]		=[b]	=	=GT	=	=	=GT	[14]
Mstn	=[b]		=[c]		↑GT	↓		↑GT	[77]
Injectable proteins									
JA16 antibody	↑	↑size =number =type	=						[5,36]
ACVR2B/Fc 4 weeks	↑	↑size =number	=	=		=	↑		[66]
ACVR2B/Fc 10 weeks			↓	↑		↓	↑		[66]
ACVR2B/Fc-CHO cells in *nude* mice	↑		↑						[65]
Myostatin	=/↓[d]		=/↓[e]						[63,64]
Myostatin in *Mstn*$^{Ln/Ln}$	=[b]					=[b]	↓		[13]
Myostatin-CHO cells in *nude* mice	↓	↓size	↓						[63]
GENETIC OBESITY									

	MUSCLE		STANDARD DIET			HIGH-FAT DIET/GENETIC OBESITY			
	Muscle mass	**Fiber Phenotype**	**Fat Mass**	**Ins sens[a]**	**Other**	**Obesity**	**Ins sens[a]**	**Other**	**References**
***MSTN* deletion**									
Mstn$^{-/-}$, *ob/ob*	↑					↓*f*		↓Hg*f*	[11]
Mstn$^{-/-}$, *A*^{y}*/a*	↑					↓		↑GT	[11]
Injectable proteins									
JA16 antibody in *db/db* mice	↑					=			[64]
PF-879 antibody in *ob/ob* mice	↑	↑size =type				=	=	↑↑	[34]

Blank, not determined; DN, dominant negative; FG, fast glycolytic fiber type; GT, glucose tolerance; Hg, hyperglycemia; Ins Sens, insulin sensitivity

[a] Insulin tolerance or glucose infusion rate during hyperinsulinemic-euglycemic clamp

[b] Measured by body composition

[c] Decreased cell size but normal body composition

[d] Decreased mass only at highest dose

[e] Published results contradict each other

[f] Young adult mice